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Impairment of the photosynthetic apparatus by oxidative stress induced by photosensitization reaction of protoporphyrin IX

Baishnab C. Tripathy *, Anasuya Mohapatra, Illora Gupta

School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

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Abstract

Treatment with the herbicide acifluorfen-sodium (AF-Na), an inhibitor of protoporphyrinogen oxidase, caused an accumulation of protoporphyrin IX (Proto IX) , light-induced necrotic spots on the cucumber cotyledon within 12–24 h, and photobleaching after 48–72 h of light exposure. Proto IX-sensitized and singlet oxygen ($^1O_2$)-mediated oxidative stress caused by AF-Na treatment impaired photosystem I (PSI), photosystem II (PSII) and whole chain electron transport reactions. As compared to controls, the F_v/F_m (variable to maximal chlorophyll a fluorescence) ratio of treated samples was reduced. The PSII electron donor NH_2OH failed to restore the F_v/F_m ratio suggesting that the reduction of F_v/F_m reflects the loss of reaction center functions. This explanation is further supported by the practically near-similar loss of PSI and PSII activities. As revealed from the light saturation curve (rate of oxygen evolution as a function of light intensity), the reduction of PSII activity was both due to the reduction in the quantum yield at limiting light intensities and impairment of light-saturated electron transport. In treated cotyledons both the Q (due to recombination of Q_A^- with S_2) and B (due to recombination of Q_B^- with S_2/S_3) band of thermoluminescence decreased by 50% suggesting a loss of active PSII reaction centers. In both the control and treated samples, the thermoluminescence yield of B band exhibited a periodicity of 4 suggesting normal functioning of the S states in centers that were still active. The low temperature (77 K) fluorescence emission spectra revealed that the F_{695} band (that originates in CP-47) increased probably due to reduced energy transfer from the CP47 to the reaction center. These demonstrated an overall damage to the PSI and PSII reaction centers by $^1O_2$ produced in response to photosensitization reaction of protoporphyrin IX in AF-Na-treated cucumber seedlings.

Keywords: Acifluorfen; Chlorophyll biosynthesis; Chlorophyll fluorescence; *Cucumis sativus*; Diphenyl ether herbicide; Oxidative stress; Photodynamic reaction; Photosystem I; Photosystem II; Protoporphyrin IX; Singlet oxygen; Thermoluminescence

1. Introduction

Accumulation of excess porphyrins, as in the case of people suffering from porphyria, or in plants treated with photodynamic herbicides, results in oxidative stress. Over-accumulated tetrapyrrolic photosensitizers, upon absorption of light, produce triplet excited states which may interact with molecular oxygen to generate singlet oxygen ($^1O_2$) via type II photosensitization reaction [1–3]. This highly reactive form of oxygen is capable of oxidizing electron-rich substrates of biological importance such as lipids and proteins to give peroxides or other oxidized products ultimately resulting in serious damage to the tissue [4,5].

The biosynthetic pathway of tetrapyrrolic intermediates protoporphyrin IX (Proto IX), Mg-protoporphyrin IX monoester (MPE) and protochlorophyllide (Pchlide) is tightly regulated. Pchlide and heme act as feedback inhibitors of biosynthesis of 5-aminolevulinic acid (ALA), the precursor of porphyrins [6,7]. Foliar application of ALA to green plants bypasses the regulatory feedback inhibition of the Pchlide pool and induces excess accumulation of Mg-tetrapyrroles [8–12]. ALA-induced accumulation of non-phototransformable Pchlide, in the presence of light, generates $^1O_2$ through type II photosensitization reaction, which damages the plant [13,14].

Abbreviations: AF-Na, acifluorfen-sodium; ALA, 5-aminolevulinic acid; DCIP, 2,6-dichlorophenol indophenol; DCMU, 3-(3,4-dichlorophenyl) 1,1-dimethyl urea; HEAR, hexane extracted acetone residue solvent mixture; MPE, Mg-protoporphyrin IX monoester; MV, methylviologen; Pchlide, protochlorophyllide; PD, *p*-phenylenediamine; Proto IX, protoporphyrin IX; RNO, *N*,*N*-dimethyl-*p*-nitosoaniline; TL, thermoluminescence

* Corresponding author. Fax: +91 11 26717558.
E-mail address: bctripathy@mail.jnu.ac.in (B.C. Tripathy).

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When ALA-treated and dark-incubated wheat and lettuce plants were exposed to continuous light, the fluidity of thylakoid membranes decreased due to photodynamic damage which coincided with an increase in $P700^+$ formation [15].

Treatment with diphenylether (DPE) and allied herbicide leads to the inhibition of protoporphyrinogen oxidase and light-induced excess accumulation of the photosensitizer Proto IX [3,12,16–18]. Definite proof for the involvement of tetrapyrroles in DPE mode of action came from the observation that inhibition of biosynthesis of ALA, the precursor of tetrapyrroles, by gabaculine in DPE-treated plants led to non-accumulation of tetrapyrroles and consequent nullification of the herbicidal activity [19]. In addition to the inhibitors of chlorophyll (Chl) biosynthesis, genetic defects in biosynthetic and degradative pathways of Chl also generate singlet oxygen and cause photodamage [20–25].

Blazer is a pre-emergent commercial herbicide that damages the photosynthetic apparatus and causes photo-bleaching of leaves leading to plant death. In the present study we show that treatment of the technical grade material of AF-Na, an active ingredient of the commercial herbicide Blazer, leads to over-accumulation of Proto IX that absorb sunlight to produce triplet excited states which interact with molecular oxygen via type II photosensitization reaction to generate singlet oxygen. The latter damages the structure and function of both PSI and PSII reaction centers resulting in the impairment of the photosynthetic apparatus.

2. Materials and methods

2.1. Plant material

Cucumber (*Cucumis sativus* L. cv Poinsette) seedlings were grown for 5 days on moist germination paper in Petri plates (14.5 cm diameter), under continuous cool white fluorescent light (75 μmol photons m^{-2} s^{-1}) at 25 °C.

2.2. AF-Na treatment

The technical grade material of AF-Na (98% pure) was a gift from Rohm and Haas Bayport Inc. USA. Technical grade AF-Na was diluted with distilled water containing 0.5% Tween-20. Petri plate having 25 seedlings was sprayed with 10 ml of 3 μM AF-Na. Control seedlings were sprayed with 0.5% Tween-20 dissolved in distilled water. Seedlings were covered with aluminum foil and kept in darkness for 14 h and exposed to cool-white fluorescent light (75 μmol photons m^{-2} s^{-1}) at 25 °C. Light intensity was measured using LI-COR Quantum meter Model LI-185B with a LI-190SB quantum sensor (LI-COR Inc., Lincoln, USA).

2.3. Pigment estimation

Chl and carotenoid contents were estimated in 80% acetone as described elsewhere [26,27].

2.4. Protoporphyrin IX determination

Homogenates of control and AF-Na-treated cucumber cotyledons in 90% chilled ammonical acetone (acetone:0.1 N NH_4OH = 9:1) (10 ml) were centrifuged in Sorvall centrifuge at 12,000×*g* for 10 min at 4 °C. The supernatant was mixed in a separating funnel with equal volume of ice-cold hexane added to it. This was mixed properly and the two layers were allowed to separate. The lower layer was reextracted with 1/3 volume of hexane. The extract i.e., the hexane-extracted acetone-residue solvent mixture (HEAR) was then used to measure fluorescence emission spectra. Fluorescence emission spectra were recorded at room temperature in a photon-counting SLM 8000 spectrofluorometer in the ratio mode. A tetraphenylbutadiene block was used to adjust the voltage to 20,000 counts per second in both the reference and the sample channels, when excited at 348 nm and the emission monitored at 422 nm. The slit widths of both the excitation and emission monochromators were set at 4 nm. Rhodamine B was used as a quantum counter in the reference channel. HEAR was placed in the sample chamber and fluorescence emission spectra were recorded from 580 nm to 700 nm. Samples were excited at 400 nm (E400), and 440 nm (E440) and fluorescence emission spectra were corrected for instrument response. Proto IX contents were calculated from fluorescence emission spectra (E400 and E440) as described elsewhere [28].

2.5. Isolation of thylakoid membranes

Thylakoid membranes were isolated from cucumber cotyledons at 4 °C, under green light, by hand-homogenizing the tissue in an isolation medium consisting of 0.4 M sucrose, 10 mM NaCl and 25 mM HEPES–NaOH (pH 7.6) [14]. The homogenate was passed through 2 layers of Mira cloth and was centrifuged at 3000×*g* for 5 min to sediment the thylakoid membranes. Thylakoids were suspended in isolation buffer.

2.6. Singlet oxygen determination

The production of $^1O_2$ was determined in terms of *N*,*N*-dimethyl p-nitosoaniline (RNO) bleaching using histidine as a trap of $^1O_2$ [14]. Thylakoids (containing 100 μg Chl ml^{-1}) were incubated in 1 ml of RNO solution (300 μM RNO in 10 mM histidine) in one tube in the dark, whereas another tube was illuminated with light (500 μmol photons m^{-2} s^{-1} obtained from a tungsten light source) for different time periods during the assay. Samples were centrifuged (10,000×*g*) after dark or light treatment and their absorbance read at 440 nm in a spectrophotometer (Shimadzu, UV160). The dark samples were used as reference.

2.7. Electron transport

Assays of electron transport activity of whole chain, PSII and PSI were carried out using a glass cuvette fitted within a Clark-type oxygen electrode (YSI,

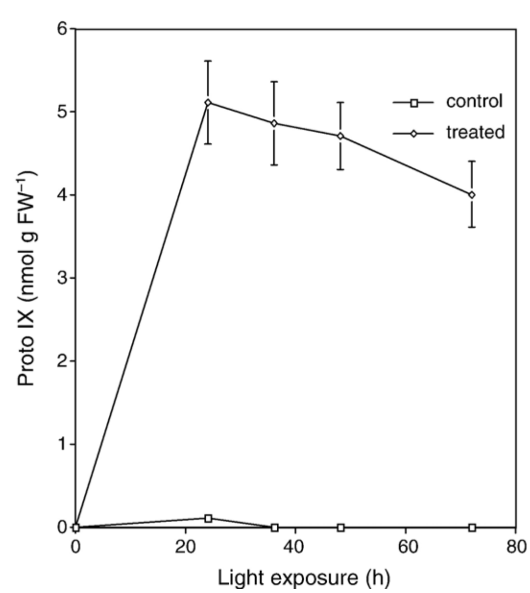

Fig. 1. Proto IX synthesis in control and AF-Na-treated seedlings exposed to light (75 μmol photons m^{-2} s^{-1}). Error bars represent SD of 3 replicates and missing error bars indicate that they are smaller than the symbol.

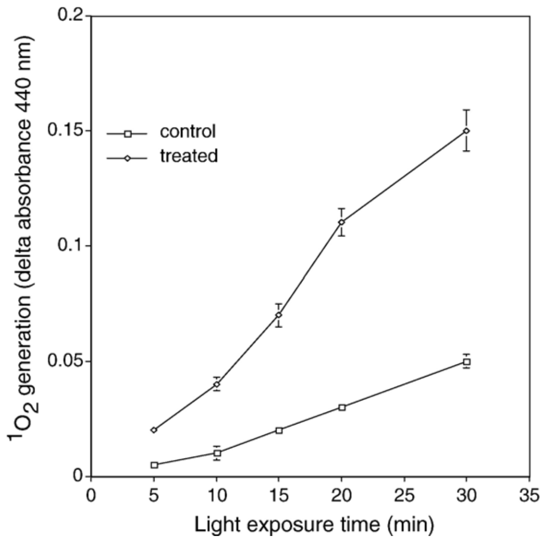

Fig. 2. RNO bleaching reactions of thylakoids isolated from control and AF-Na -treated seedlings exposed to cool-white fluorescent light (75 μmol photons m^{-2} s^{-1}) for 2 h. Error bars represent SD of 3 replicates and missing error bars indicate that they are smaller than the symbol.

Yellow springs, USA). The reaction mixture was maintained at 25 °C by using a temperature controlled water bath and was illuminated for 20 s with white light from a tungsten light source at a photon flux rate of 1500 μmol m^{-2} s^{-1}. The whole chain electron transport from H_2O to methylviologen (MV) (1 mM) was monitored as O_2 uptake. PSII activity was monitored as O_2 evolution from H_20 to *p*-phenylenediamine (PD) (0.5 mM). The partial electron transport chain through PSI was measured as oxygen consumption. Ascorbate (1 mM)/ DCIP (0.1 mM) couple was used as electron donor to PSI and MV (1 mM) was used as electron acceptor [29]. Electron flow from PSII was blocked by 3-(3,4-dichlorophenyl) 1,1-dimethyl urea (DCMU) (20 μM).

2.8. Light saturation curve

Light saturation curve of PSII dependent oxygen evolution was measured at various light intensities. Maximum white light intensity (100% saturation) was 1500 μmol photons m^{-2} s^{-1}, obtained from a tungsten light source. Various lower light intensities were obtained using neutral density filters (Blazers).

2.9. Chlorophyll a fluorescence transient

Fluorescence transients of thylakoids were monitored by pulse amplitude modulated (PAM) Chl fluorometer (PAM 101, Heinz Walz, Germany). For measurement of initial F_0, modulated light of 1.6 KHz (2.5 μmol photons m^{-2} s^{-1}) was used. For measurement of F_m, modulated light of 100 KHz along with actinic light (900 μmol photons m^{-2} s^{-1}) was used while keeping the other settings same as that for F_0 measurement. Isolated thylakoids at a concentration of 10 μg Chl ml^{-1}, suspended in a buffer containing 50 mM sucrose and 10 mM HEPES (pH 7.6) were dark adapted for 20 min before measurements.

2.10. Chlorophyll a fluorescence spectra

Room temperature (25 °C) and low temperature (77 K) fluorescence emission spectra of thylakoids were recorded in ratio mode in a photon counting SLM 8000 spectrofluorometer. Rhodamine B was used in the reference channel as a quantum counter. A tetraphenylbutadiene block was used to adjust the voltage in the sample as well as in the reference channels to 20,000 counts s^{-1} at excitation and emission wavelengths of 348 and 422 nm, respectively. The excitation and emission slit widths were 4 nm. For room temperature emission spectra, thylakoids, containing 3 μg Chl ml^{-1}, were suspended in 10 mM HEPES–NaOH buffer (pH 7.6). For 77 K Chl a emission spectra, thylakoids equivalent to 2 μg Chl ml^{-1} were suspended in 10 mM HEPES–NaOH buffer (pH 7.6) containing 25% glycerol. The spectra were corrected for the instrument response [28].

2.11. Thermoluminescence

Leaf discs of 0.5 cm in diameter were punched from cotyledons of untreated or AF-Na-treated cucumber seedlings exposed to light. The discs were placed in a stainless steel planchet and quickly frozen to 77 K by dipping the planchet in liquid nitrogen. DCMU-treatment of leaf was achieved by vacuum infiltration of 10 μM DCMU. The planchet containing frozen leaf discs was transferred to the cryostat previously cooled to 77 K [30]. The leaf disc was heated in dark by warming the planchet holder by a temperature controller programmer. Thermoluminescence (TL) emanating from the leaf, as the temperature was increased by heating the planchet, was monitored by an EMI 9558 QB photomultiplier tube having S-20 response. The signal was amplified and was fed to one X–Y chart recorder. The temperature of the leaf was monitored by an iron-constantan thermocouple placed below the planchet. For measurements on flash number dependent TL, leaf discs were dark-incubated for 20 min and then illuminated with a sequence of 3 μs, 0.5 J white-light flashes and quickly frozen to 77 K [31,32].

3. Results

There was no difference between controls and AF-Na-treated seedlings kept in dark for 72 h. The effects of the herbicidal treatment were observed only in light. AF-Na-treated seedlings incubated in dark for 14 h, and subsequently transferred to cool white fluorescent light (75 μmol photons m^{-2} s^{-1}), developed

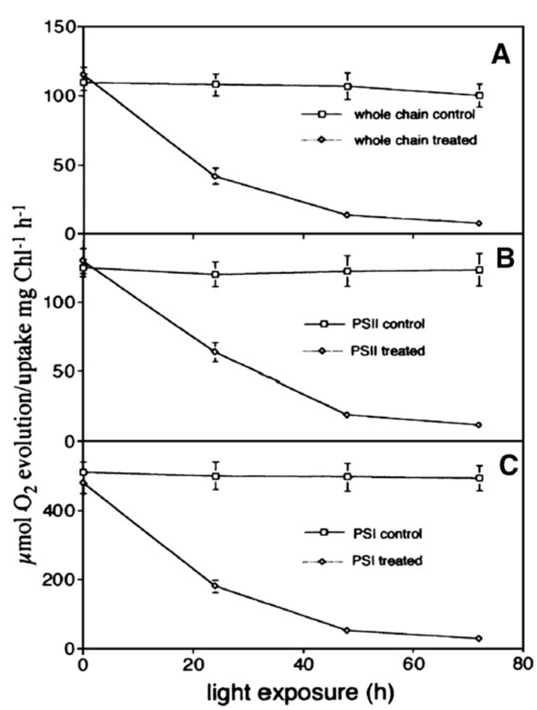

Fig. 3. Impairment of photosynthetic electron transport chain by the herbicide AF-Na. AF-Na-sprayed cucumber seedlings were incubated in dark for 14 h and subsequently exposed to cool-white fluorescent light (75 μmol photons m^{-2} s^{-1}) for 72 h. Thylakoids were isolated from cotyledons harvested at desired time intervals and their photochemical functions were monitored as described in Materials and methods. Each data point is the average of 3 replicates and error bars represent SD. Missing error bars indicate that they are smaller than the symbols.

Table 1
Effect of exogenous PS II electron donor NH_2OH (10 mM) on background fluorescence (F_0), maximum fluorescence (F_m), variable fluorescence (F_v) and F_v/F_m ratio of thylakoids isolated from control and AF-Na-treated cucumber seedlings, exposed to 24 h of light (75 μmol m^{-2} s^{-1})

Sample	Electron donor	F_0	F_m	F_v	F_v/F_m
control	H_2O	2.0±0.1	9.5±0.5	7.5±0.6	0.79±0.08
control	NH_2OH	2.3±0.2	9.6±0.5	7.3±0.6	0.76±0.09
AF-Na-treated	H2O	2.9±0.2	6.6±0.5	3.7±0.4	0.56±0.05
AF-Na-treated	NH_2OH	3.0±0.3	6.7±0.5	3.7±0.4	0.55±0.07

Thylakoids (10 μg Chl mL^{-1}) were used for measuring fluorescence transients on 'PAM Chlorophyll Fluorometer'. Data points are mean of 4 replicates and ± represents SD.

necrotic spots within 12 h and were severely photo-bleached after 36–72 h (not shown). This bleaching of seedlings was not due to water stress since enough water was present in Petri plates when seedlings were exposed to light; it was caused by photodynamic events.

3.1. Pigment content

Pigment content of the AF-Na-treated seedlings, corrected for the loss of moisture content, declined upon exposure of seedlings to cool-white-fluorescent light (75 μmol photons m^{-2} s^{-1}). The total Chl content was reduced by 30% after 24 h and 61% after 72 h of light exposure. Loss of Chl *a* was greater than that of Chl *b*. Consequently, after 48 h of light exposure the Chl a/b ratio decreased by 40%. Carotenoid content in treated seedlings decreased by 32% after 24 h and 65% after 72 h of light exposure. Under identical conditions of light exposure, control seedlings did not have any loss of pigments. Thus, we interpret these changes in the pigments to be due to the treatment by AF-Na, and the photodynamic damage associated with it.

3.2. Protoporphyrin IX accumulation

In dark, Proto IX did not accumulate. However, when AF-Na-treated cucumber seedlings, incubated in dark for 12 h, were exposed to cool-white-fluorescent light (75 μmol photons m^{-2} s^{-1}) for 72 h, Proto IX accumulation increased up to 24 h, and this accumulation continued after 72 h of illumination; controls showed no increase in Proto IX (Fig. 1).

3.3. Singlet oxygen production

Tetrapyrroles are potential photosensitizers and produce singlet oxygen ($^1O_2$) via type II photosensitization reactions [1–3]. To ascertain if $^1O_2$ is produced in AF-Na-treated seedlings that accumulated Proto IX, thylakoid membranes were isolated from cotyledons of control and AF-Na-treated seedlings exposed to cool-white-fluorescent light (75 μmol photons m^{-2} s^{-1}) for 4 h and then the $^1O_2$ production was monitored by RNO bleaching reaction. Upon illumination of thylakoids $^1O_2$-mediated RNO bleaching increased more than three times in AF-Na-treated than control after 30 min of light exposure (Fig. 2).

3.4. Photochemical activities

Since both PSI and PSII are located in thylakoid any damage to thylakoid membrane is likely to affect the activities of both the photosystems. Fig. 3 shows changes in electron transport rates of thylakoid membranes isolated from 14-h dark-incubated control and AF-Na-treated seedlings exposed to light up to 72 h. A major decrease in all activities was observed by 24 h light exposure, PS I having a larger change than PSII. Between 24 and 72 h of light exposure, the electron transport rates in thylakoids isolated from AF-Na-treated seedlings declined by ~60–90% for whole chain (H_2O to MV) (Fig. 3A), ~50–90% for PSII (H_20 to PD) (Fig. 3B) and 60–95% for PSI ($DCIPH_2$ to MV) (Fig. 3C). Photochemical activities of AF-Na-treated seedlings incubated in dark were similar to those of dark-incubated control seedlings (data not shown) suggesting that light exposure was essential to induce the inhibition observed. A comparison with Fig. 1 suggests a general correlation with Proto IX formation.

3.5. Effect of exogenous electron donor NH_2OH on variable fluorescence

Chl a fluorescence is often used as a signature of photosynthesis. As shown in Table 1, the F_0 goes up from a value of 2 to 2.9, i.e., by 1.5 times. In addition to an increased F_0, a substantial reduction in F_{max} was observed that decreased from 9.5 to 6.6. This resulted in decrease in F_v/F_m ratio from

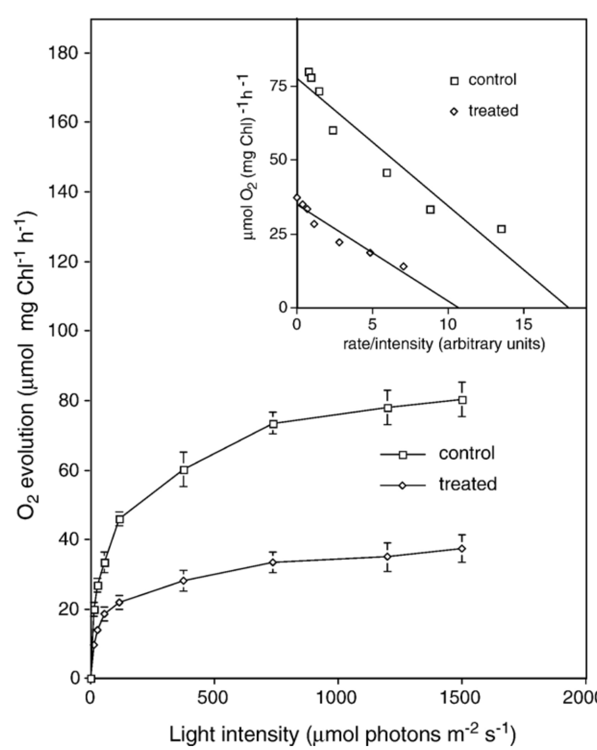

Fig. 4. The light saturation curve of PSII of thylakoid membranes isolated from control and AF-Na-treated cucumber seedlings incubated in dark for 14 h and subsequently exposed to cool-white fluorescent light (75 μmol photons m^{-2} s^{-1}) for 24 h. Each data point is the average of 3 replicates and error bars represent SD. Missing error bars indicate that they are smaller than the symbols.

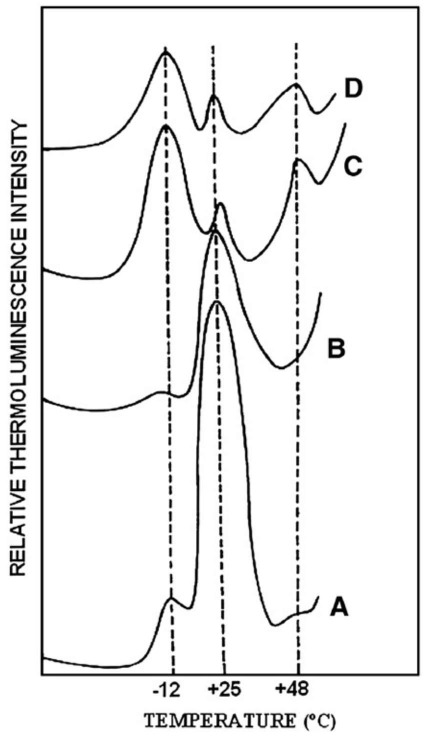

Fig. 5. TL of leaf discs taken from cotyledons of control (curve A, without DCMU; curve C, with DCMU) and AF-Na-treated (curve B, without DCMU; curve D, with DCMU) seedlings exposed to cool-white fluorescent light (75 µmol photons m^{-2} s^{-1}) for 24 h. Other experimental details were as in Materials and methods. TL curves are staggered on the *Y* axis for clarity.

0.79 in controls to 0.56 in treated samples. To investigate the nature of AF-Na-mediated oxidative damage of PSII activity, the effect of exogenous PSII electron donor NH_2OH (10 mM) [33–35] on Chl *a* fluorescence induction kinetics was measured. As compared to control, the F_v/F_m ratio of thylakoid membranes isolated from cucumber cotyledons treated with AF-Na and exposed to light for 24 h was reduced by 30% (Table 1) demonstrating the loss of PSII activity. In the presence of exogenous electron donor NH_2OH (10 mM) the F_v/F_m ratio of thylakoid membranes isolated from control samples was not affected. However, NH_2OH failed to restore the inhibited F_v/F_m ratio in treated samples to control level.

3.6. Light saturation curve

To further ascertain if the inhibition of PSII photochemical reaction was due to reduction in the quantum yield of PSII or in the light saturated electron transport rate or both, the rate of PSII reaction as a function of light intensity was measured in thylakoid membranes isolated from control and AF-Na-treated seedlings exposed to light for 24 h. Dependence of PSII activity on light intensity showed typical saturation kinetics (Fig. 4). Both the initial slope at limiting light intensities as well as light-saturated electron transport rates were affected in AF-Na-treated seedlings. As compared to control, the percent inhibition of PSII reaction in treated thylakoids was almost constant (nearly 50%) at all the light intensities used. The Eadie plot (i.e., the rate of oxygen evolution *versus* v/light intensity i.e. % saturation) [36] for control and treated (inset Fig. 4) reveals a marked drop in the quantum efficiency of PSII reaction (intercept on the abscissa) and a substantial reduction of V_{max} (intercept on the ordinate) due to oxidative stress. Both V_{max} and quantum efficiency of PSII were reduced almost by 50%. We suggest that inhibition of V_{max} and quantum efficiency of PS II was due to damage to the reaction center.

3.7. Thermoluminescence

Thermoluminescence is a measure of the back reactions of PSII [37–46]. Fig. 5 shows the TL of cucumber cotyledons in control and treated seedlings exposed to light (75 µmol photons m^{-2} s^{-1}) for 24 h. In control samples, the TL at −12 °C, 25 °C and 48 °C are due to Q, B and C bands respectively (curve A) [37–46]. When cotyledons harvested from control seedlings

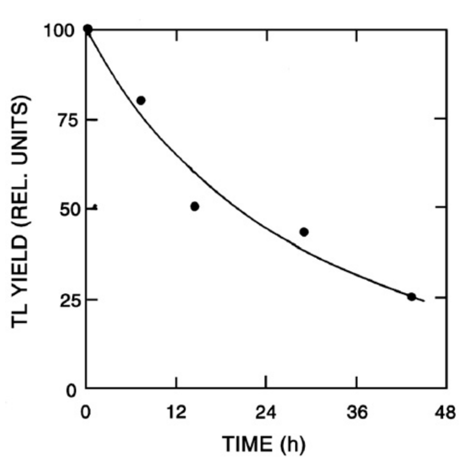

Fig. 6. Total TL yield of leaf discs taken from cotyledons of AF-Na-treated seedlings exposed to different lengths of cool-white fluorescent light (75 µmol photons m^{-2} s^{-1}).

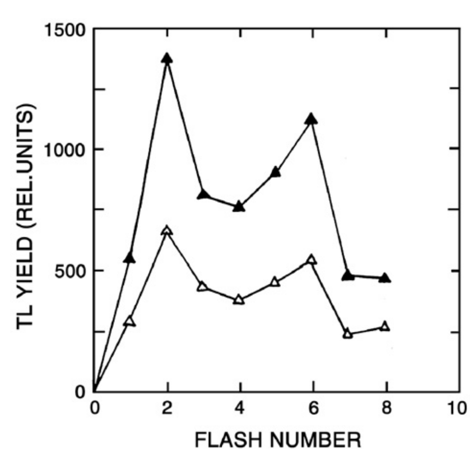

Fig. 7. The flash yield of B band of leaf discs taken from control and AF-Na-treated seedlings exposed to cool-white fluorescent light (75 µmol photons m^{-2} s^{-1}) for 24 h. Other experimental details were as in Materials and methods.

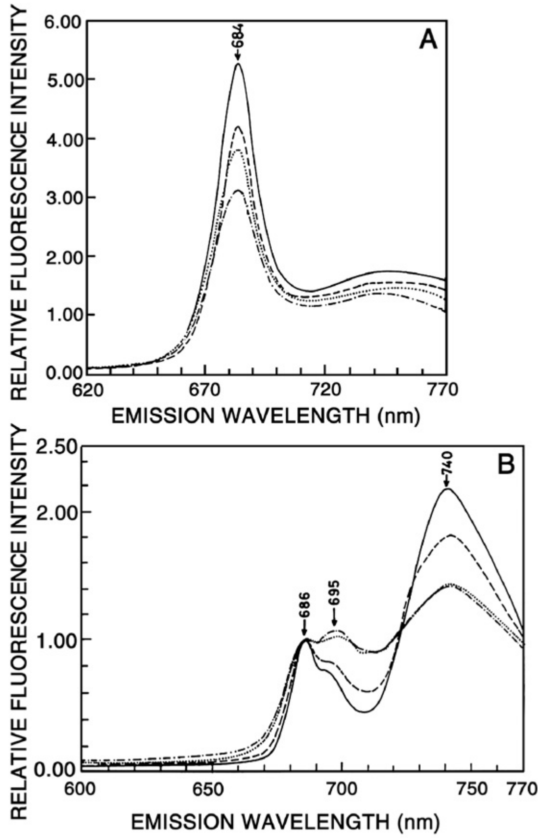

Fig. 8. Room temperature (25 °C) (A) and low temperature (77 K) fluorescence emission spectra (E440) of thylakoid membranes isolated from dark-incubated AF-Na-treated cucumber seedlings exposed to cool-white fluorescent light (75 μmol photons m^{-2} s^{-1}) for 0 h (_____), 36 h (--------), 48 h (.........) and 72 h (_._._._._). Low temperature fluorescence spectra were normalized at 686 nm. Fluorescence emission spectra of isolated thylakoid membranes were recorded in ratio mode in a photon counting SLM-AMINCO 8000 spectrofluorometer having excitation and emission slit widths of 4 nm. Spectra were corrected for the instrument response.

were vacuum infiltrated with DCMU (30 μM), that blocked electron transport from Q_A to Q_B, the intensity of Q band increased and that of B band highly diminished (curve C). A residual B band may be due to incomplete penetration of DCMU or due to the presence of some residual Q_B^- in the sample [37]. The intensity of C band also increased due to DCMU treatment (curve C). As compared to the control, in AF-Na-treated seedlings, the Q band intensity sharply decreased (curve B). When AF-Na-treated and light-exposed cotyledons were vacuum infiltrated with DCMU (curve D) the intensity of Q and C bands increased and that of B band highly diminished.

The total TL yield, measured as the area under the curve, in AF-Na-treated and light exposed samples was much less (55% at 24 h; 75% at 42 h) than that of control (Fig. 6) showing damage to the PSII reaction centers.

In order to understand if the S states are affected due to AF-Na-treatment, the flash yield of the B band was studied. As shown in Fig. 7, the TL yield was maximum on 2nd and 6th flash, having a periodicity of 4 [(cf. 43)]. In AF-Na-treated seedlings exposed to light for 24 h, the peaks for flash oscillation of TL yield of B band also demonstrated periodicity of 4 having maxima at 2nd and 6th flashes. However, the TL yield was reduced by 50% in treated samples. These data show that the residual undamaged PS II functioned normally.

3.8. Chlorophyll a fluorescence spectra

At room temperature, thylakoids isolated from control seedlings when excited at 440 nm had a peak at 684 nm emanating from PSII [47]. Although there was no distinct spectral shift, the fluorescence intensity due to PSII at 684 nm decreased progressively in thylakoids isolated from AF-Na treated seedlings exposed to light for 24 h (not shown), 36 h, 48 h and 72 h (Fig. 8A). This further confirms that the undamaged PSII antenna was normal and unchanged.

The low temperature (77 K) fluorescence spectra of thylakoid membranes isolated from control and AF-Na-treated seedlings when excited at 440 nm had emission bands at 686 nm (PS II antenna) and at 695 nm (CP47) and a large fluorescence emission at 740 nm mostly from PSI [47] AF-Na treated seedlings exposed to light for 36 h, 48 h and 72 h were normalized at 685 nm in order to observe spectral changes at other wavelengths relative to that at 686 nm (Fig. 8B). F_{695} substantially increased and was higher than F_{686} peak in AF-Na-treated seedlings exposed to light for 48 h and 72 h. Under identical conditions PSI emission at 740 nm significantly declined. This resulted in changes in peak ratios. In control samples, F_{686}/F_{695} was 1.34 that declined to 0.94 and 0.92 in treated seedlings exposed to light for 48 h and 72 h. Similarly F_{740}/F_{686} was 2.19 in control samples and it declined in treated seedlings exposed to light for 48 h and 72 h. Thylakoid membranes isolated from AF-Na-treated seedlings exposed to light for 24 h did not show any significant shifts or changes in fluorescence emission spectral position pertaining PSI or PSII (spectra not shown).

4. Discussion

AF-Na treatment results in light-induced over-accumulation of Proto IX (Fig. 1) leading to generation of $^1O_2$ (Fig. 2) via type II photosensitization reaction. This results in the impairment of the photosynthetic electron transport by affecting PSI and PSII activities (Figs. 3 and 4). As compared to AF-Na-treatment (studied here) where it takes almost 24 h to get 50% inactivation of photosynthetic reactions, in ALA-treated cucumber seedlings 50% inhibition of PSII occurs within 2 h of light exposure [2] demonstrating that ALA-mediated herbicidal action is more severe than that of AF-Na. ALA-induced oxidative stress is photosensitized by the Chl biosynthetic intermediate Pchlide [13,14] whereas that induced by AF-Na is mediated by the tetrapyrrole Proto IX [3]. $^1O_2$ is generated by both the photosensitizers i.e., Pchlide [14] and Proto IX (Fig. 2) and impairs the photosynthetic photochemical reactions ([3,14]; this paper). However, the differential extent of injury to the thylakoid membrane caused by the photosensitizer Pchlide or Proto IX is probably due to their differential localization in the cell. Pchlide is mostly bound to thylakoid membrane, whereas Proto IX is mostly localized in the soluble phase although it also partially partitions itself to thylakoids [3]. $^{1}O_2$ generated by the thylakoid membrane-localized photosensitizer Pchlide could immediately penetrate the membrane and cause quick damage to the photosynthetic apparatus. On the other hand, $^{1}O_2$ produced from Proto IX, is likely to be significantly quenched in the aqueous phase before they reach the membranes. Therefore, AF-Na-induced oxidative stress is probably slower than that of ALA.

The ratio of F_v/F_m is a measure of the functional status of PSII. Increase in F_0 due to AF-Na treatment, could be due to decreased excitation energy transfer from the antenna to PSII reaction center. This itself will lead to lowered PSII reaction center activity (Figs. 3 and 4) and increase in PSII antenna (F_{695}) fluorescence (Fig. 8B). The exogenous electron donor NH_2OH failed to restore the F_v/F_m ratio in AF-Na-treated seedlings. High concentration of NH_2OH, used in this work, that is known to remove the Mn cluster and to act as an electron donor to PSII [33–35], restores the variable fluorescence to normal level. Thus the loss of F_v/F_m in AF-Na-treated samples relative to that in the untreated samples reflects the loss of reaction center function. The identical F_v/F_m values obtained without and with NH_2OH treatment in the AF-Na-treated samples actually demonstrates that there is no specific donor-side damage. This explanation is supported by the near-similar loss of PSI and PSII activities. This demonstrates an overall damage to both the PSI and PSII reaction centers by the $^{1}O_2$ produced from protoporphyrin IX-mediated type II photosensitization reactions. TL [38] is a useful probe of back reaction of PSII [39,41]. The TL bands at −12 °C, 25 °C and 48 °C are due to Q, B and C bands respectively The Q (D) band is due to charge recombination of the primary quinone acceptor Q_A^- and S2/3 states whereas the B band arises due to charge recombination of the secondary quinone acceptor Q_B^- with S2/3 states and the C band emanates from charge recombination of Q_A^- S states namely S1 [43,44]. Although, the distance between the peaks is not precisely proportional to temperature difference since heating rate was not constant in our experiments, yet we can quantitate the peak and intensity of TL. The intensification of Q band by the addition of DCMU is accompanied by the loss of B band and increase of C band [29,42]. As stated earlier, a residual B band, observed in the presence of DCMU, is due to its incomplete penetration into the leaves and/or due to presence of Q_B^- in dark-adapted leaves [37]. In cotyledons, harvested from light exposed AF-Na-treated seedlings, the B band intensity sharply decreased by around 50% and Q and C bands also declined by 50% demonstrating uniform 50% damage to PSII reaction centers by Proto IX-sensitized oxidative damage. The characteristic oscillation of TL band is affected when water oxidation is damaged, for example, when 33 kDa protein involved in water oxidation is removed [46]. In both the control and the treated samples, the TL yield observed a periodicity of 4 [45] and the maximum yield were obtained on 2nd and 6th flashes which were similar to earlier report [30,37]. This shows that non-damaged PSII centers behave normally. There was no significant change in any of the temperature maxima of the various bands suggesting no change in the activation energies of the various back reactions of PS II [43].

The reduction in fluorescence peak height measured at room temperature (when excited with an actinic light beam having a bandwidth of 16 nm) was due to the loss of variable fluorescence in the treated samples (Fig. 8A). This confirms the earlier observations that pertubation of thylakoid membrane [29,33,48] leads to changes in Chl a fluorescence properties. Gross perturbation of the thylakoid membrane usually induces a spectral shift in the Chl *a* fluorescence spectra of chloroplasts and leaves at low temperature (77 K). The low temperature fluorescence spectra have peaks at 685 nm (F_{685}) and at 695 nm (F_{695}) emissions, which mostly originate from the PSII CP 43 and CP 47 respectively [47,49] and a large F_{735} peak that originates mostly from PSI [50]. If LHCPI is removed from PSI by detergent treatment, the inner antenna of reaction center I (RCI) fluoresces at 722 nm [51]. Isolated LHCI fluoresces around 735 nm [52]. Thus it is apparent that inner antenna of RCI emits F_{722} and LHCI emits F_{735} [53]. The relative increase in F_{695} emission (Fig. 8B) in thylakoids isolated from AF-Na-treated seedlings exposed to light for 36–72 h might be partly due to functional detachment of CP47 from the D1/D2 reaction center which would have resulted in reduced energy transfer to the reaction center and consequent relative increase in CP47 fluorescence. It is one of a few examples where CP 47 fluorescence (F_{695}) increases relative to that of CP43 (F_{685}) and demonstrates the dual origin of F_{686} and F_{695} fluorescence peaks of PSII. The relative decline in F_{740} may be due to either reduced energy transfer to PSI from PS II antenna and/or due to damage to LHCI induced by Proto IX-sensitized oxidative reactions. This is in contrast to ALA-mediated Pchlide-sensitized oxidative stress [13] where the ratio of F_{686}/F_{695} almost remained the same in both the control and the treated samples and the fluorescence peak due to PSI declined and blue-shifted from 735 nm to 728 nm suggesting disorganisation of LHCI.

In conclusion, $^{1}O_2$ generated *via* type II photosensitization reaction of Proto IX in light-exposed AF-Na-treated cucumber seedlings affects the structure and function of the photosynthetic apparatus leading to overall inactivation PSI and PSII reaction centers.

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